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Analysis of potyvirus terminal protein VPg-transgenic *Arabidopsis thaliana* plants

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Virus-coded VPg protein of *Potato virus Y* (PVY) does not have homologs apart from other VPgs. Since VPg is indispensable for the potyvirus life cycle, it appeared a good candidate for eliciting pathogen-derived resistance to PVY. Following agroinfection used to obtain PVY VPg-transgenic *Arabidopsis thaliana* plants, only few transgenic seeds were recovered giving rise to six transgenic plants that contained the *VPg* gene with the correct sequence. They generated VPg mRNA, but VPg protein was not detected. Some plants were immune to PVY infection suggesting post-transcriptional gene silencing. However, the likely PVY VPg toxicity exerted at an early stage of transformed seeds development precludes its use for engineering pathogen-derived resistance.

Keywords: Potyvirus, VPg protein, transgenic plant, pathogen-derived resistance

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INTRODUCTION

Potyviruses (genus *Potyvirus*) represent the largest genus of aphid-transmitted plant-infecting viruses that induce harmful diseases in dicot and monocot crop plants. They have a single-stranded messenger-polarity RNA genome of about 10 000 nucleotides, a poly(A) tail at the 3' end, and the a VPg protein covalently attached to the 5' terminus. The viral RNA contains a single open reading frame encoding a polyprotein that is cleaved into functional proteins by virus-encoded proteinases (Rajamäki *et al.*, 2004). One of the viral proteins, VPg, is a well-known virulence factor. Indeed, the covalent tyrosine residue-mediated linkage between VPg and viral RNA is required for potyvirus infectivity; mutation of the tyrosine residue abolishes viral replication (Murphy *et al.*, 1996). In addition, treatment of virus RNA with proteinase K that digests VPg significantly reduces infectivity (Redinbaugh *et al.*, 2001). At the beginning of polyprotein processing VPg exists as a fusion protein NIa, also called VPg-Pro, and is liberated in a free form after autocatalytic cleavage by the Pro protease.

VPg is responsible for numerous important virus-host interactions during the viral infection cycle. It seems to be involved in protein synthesis and cell-to-cell and long-distance movement in plant and is a determinant of potyvirus systemic infection in certain host plants (Schaad *et al.*, 1997; Lellis *et al.*, 2002; Rajamaki & Valkonen, 2002; Dunoyer *et al.*, 2004). Additionally, VPg protein interacts with potyviral RNA polymerase NIb and is involved in genome replication (Schaad *et al.*, 1996; Fellers *et al.*, 1998; Puustinen & Mäkinen, 2004). Furthermore, it has been found that PVA VPg can be phosphorylated by plant kinases (Ivanov *et al.*, 2001); it was hypothesized that this could trigger the disassembly of infecting virions and the subsequent initiation of potyvirus protein synthesis in the infected cell (Puustinen *et al.*, 2002). The PPV VPg has been shown to interact with two DEAD-box helicase-like proteins from peach and *Arabidopsis,* which interaction is necessary for the infection with PPV and also with TuMV (Huang *et al.*, 2010). Finally, the PVA VPg interacts with fibrillarin, a nucleolar protein, whose depletion reduces accumulation of infectious virons (Rajamäki & Valkonen, 2009). Several studies have reported on an interaction between the potyvirus VPg protein and the eukaryotic translation initiation factor eIF4E, which is an mRNA 5 cap-binding protein (Wittmann *et al.*, 1997; Leonard *et al.*, 2000; Duprat *et al.*, 2002; Kang *et al.*, 2005; Grzela *et al.*, 2006; Khan *et al.*, 2008). The key role of this interaction in the virus life cycle is supported by observations that mutations in either VPg or in eIF4E that abolish this interaction prevent viral infection in planta (Leonard *et al.*, 2000; Duprat *et al.*, 2002; Ruffel *et al.*, 2002; Hwang *et al.*, 2009). All these data underscore the crucial role of VPg in the virus life cycle.

Pathogen-derived resistance (PDR) is a powerful strategy to engineer plant resistance to RNA viruses (Ming *et al.*, 2008, and references therein). It is based on protein-mediated resistance (Powell-Abel *et al.*, 1986; Bendahmane *et al.*, 2007) or, more often, on RNA silencing-mediated resistance, known as posttranscriptional gene silencing (PTGS) (Van den Boogaart *et al.*, 2004; Lindbo & Dougherty, 2005; Voinnet, 2005; Savenkov & Valkonen, 2002). In plants, PTGS is a gene-regulatory mechanism involved in several control processes, including development, maintenance of genome stability, and defence against invasive pathogens (Matthew, 2004). This process is initiated by double-stranded (dsRNA) or hairpin RNA molecules (Vargas *et al.*, 2008) and results in degradation of cognate cytoplasmic or viral mRNA (Sijen & Kooter 2000; Aravin *et al.*, 2002; Hammond *et al.*, 2001). Dur-

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Abbreviations: A. t., *Arabidopsis thaliana* (*A. thaliana*); dpi, days post inoculation; eIF4E, eukaryotic initiation factor 4E; NIa, nuclear inclusion protein a; PPV, *Plum pox potyvirus;* PVA, *Potato virus A;* PVY, *Potato virus Y;* TuMV, *Turnip mosaic virus;* VPg, genome linked viral protein; wt, wild type

ing silencing, the dsRNA is processed to short interfering RNAs (siRNA) of 21–25 nucleotides by an RNase III-like enzyme called Dicer (Ramachandran & Chen, 2008). These siRNAs confer specificity on the endonuclease-contning, RNA-induced silencing complex (RISC), which targets homologous RNAs for degradation. It has been well established that PTGS is a major plant defense response against viruses (Ding & Voinnet, 2007) and viruses have been described as activators as well as targets of this mechanism (Ding *et al*., 2004; Wang & Metzlaff, 2005).

Many examples of virus resistance and posttranscriptional gene silencing of endogenous or reporter genes have been described in transgenic plants containing transgenes. The PTGS mechanism is typified by the highly specific degradation of both the transgene mRNA and the target RNA, which contains either the same or complementary nucleotide sequences. The endogenous RNA-dependent RNA polymerase (RdRp) may synthesize dsRNA using the transgene mRNA as a template, resulting in the activation of transgene silencing. If the transgene contains viral sequences, then the viral genomic RNA containing these sequences cannot accumulate in the plant (Waterhouse *et al*., 1998). Alternatively, transgene mRNA may hybridize to the viral (−)RNA strand (replicative form) to induce silencing, but this situation requires infection and accumulation of a threshold level of the viral RNA. Consequently, transgenic plants expressing virus-derived transgenes may by initially susceptible to homologous viruses and show typical infection symptoms, but new leaves developed later are symptom- and virus-free and resistant to subsequent infections with the same virus (Ratcliff *et al*., 1997; Hamilton *et al*., 2002; Savenkov & Valkonen, 2002; Yoo *et al*., 2004).

We have shown that the VPg protein of PVY is an intrinsically unfolded protein (Grzela *et al*., 2008), which supports the notion of its multifunctionality and explains one of the mechanisms for its multiple interactions with both viral and host partners. It has been demonstrated that native structural disorder is a weak signal for protein degradation and, in consequence, such proteins have a rather long half-life (Tompa *et al*., 2008). In addition, PVY VPg does not seem to have any homologs apart from other VPgs, as shown by thorough database screening (P. Siedlecki, personal communication). All these features could be important for pathogen-derived resistance (PDR) based on protein-mediated resistance. Finally and most importantly, this protein is indispensable for successful viral infection; therefore, a mechanism preventing accumulation of VPg mRNA during PVY infection could be useful for engineering plant immunity. For all these reasons we attempted to engineer *A. thaliana* plants expressing VPg of *Potato virus Y*. We then analyzed the effect of the VPg transgene on growth, development and immunity of the transgenic plants.

METHODS AND RESULTS

Arabidopsis thaliana VPg-transgenic plants were obtained by agroinfection. For this purpose, *VPg* gene (544 nt) from *Potato virus Y* (PVY strain 0, accession no. Z29526, British isolate) was amplified by PCR using plasmid pGEX-4T-1_VPg (prepared in our group) as template, digested by *Bam*HI and *Xba*I and inserted into the binary pFP101 vector under the control of the double enhanced cauliflower mosaic virus (CaMV) 35S promoter, yielding plasmid pFP101VPg. This vector contains GFP under the control of the At2S3 seed-specific promoter (Bensmihen *et al*., 2004). GFP expression allows selection of transgenic *Arabidopsis* seeds on the basis of their fluorescence, without using any antibiotic or herbicide. *Agrobacterium tumefaciens* (ASE strain) was transformed with pFP101VPg plasmid in the following way. Competent cells were prepared from *A. tumifaciens* grown at 30 °C in LB medium with rifampicin (50 μg/ml) and gentamycin (20 μg/ml), by a standard $CaCl_2$ method. Competent *A. tumifaciens* were transformed with pFPVPg (1 μg) and plated on LB agar containing rifampicin, gentamycin and spectinomycin (70 μg/ml). The presence of the *VPg* gene was demonstrated by cleavage with *Pst*I restriction endonuclease of plasmids purified by a standard method. Recombinant *A. tumefaciens* was used to transform wild-type *A. thaliana* (ecotype Columbia) by the floral dip method (Fraley *et al*., 1985).

The first experiment generated only one transformed seed. For the second inoculation developmentally younger plants were used, which resulted in nine transformed seeds (F1 progeny). Since in a parallel experiment with the same plant and vector but with a different gene about 100 seeds were obtained, the low number of VPg-transgenic seeds recovered suggested VPg toxicity exerted at an early stage of transformed seed formation.

Transformed F1 seeds were planted and grown for greenhouse experiments under natural daylight supplemented with illumination by sodium halide lamps (photoperiod 16 h, temperature 20/25 °C at night/day). Only six plants were recovered (designated F1_At_VPg1–6 lines), all morphologically similar to wt *A. thaliana*. However, in comparison with wild type plants, the transgenic seedlings grew rather slowly. Seeds (F2 progeny) from each of the F1_At_VPg lines were collected and planted. The F2 progeny, designated F2_At_VPg lines, were screened by RT-PCR and Western blotting for the presence of the VPg transgene and to of the VPg protein. Total RNA was extracted from leaves of 4-week-old seedlings using the Trizol method (http://www.umich.edu/~caparray/PDF/Trizol.pdf), treated carefully with DNase (Promega, Madison, USA) and checked for contamination with genomic DNA by PCR amplification using *Taq* polymerase (Fermentas). Subsequently, cDNA was synthesized by RT-PCR with an oligo dT(16) primer

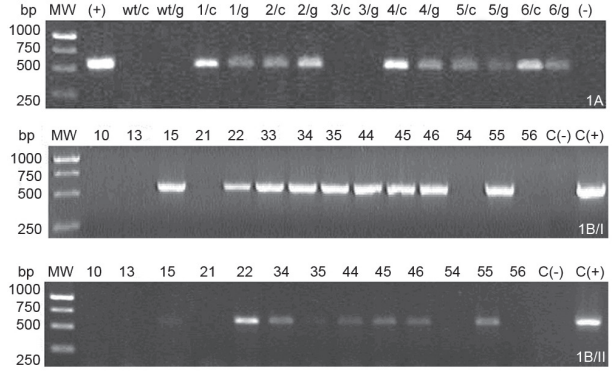

Figure 1. RT-PCR analysis of F2_At_VPg and F3_At_VPg lines with primers specific for the *VPg* gene
(1A) 1 to 6, F2_At_VPg lines; cDNA (c) and genomic DNA (g) as templates, wt, wild type; (+), positive control (pGEX-4T-1_VPg plasmid); (–), negative control (no template). **(1B)** 10–56 selected plants from F3_At_VPg lines (see Table 1), cDNA (panel **I**) or genomic DNA (panel **II**) as templates, C(+), positive control (pGEX-4T-1_VPg plasmid); C(–), wt *A. thaliana* DNA, MW, DNA molecular weight markers.

Table 1. F3 progeny plants selected for molecular analysis
Plants with transgene expression are marked in bold.

F3_At_VPg lines	Selected plants
F3_At_VPg1	10, 13, **15**
F3_At_VPg2	21, **22**
F3_At_VPg4	**33, 34, 35**
F3_At_VPg5	**44, 45, 46**
F3_At_VPg6	54, **55**, 56

and MMLV reverse transcriptase (Promega, Madison, USA). PVY-specific primers were designed according to the sequence of PVY. RT-PCR of *A. thaliana* actin mRNA was used as an internal control. Western analyses were performed as described by Sambrook *et al.* (2000) using anti-VPg rabbit polyclonal antibody (prepared in Elevage Scientifique des Dombes in Chatillon, France, using as antigen baculovirus-expressed His-VPg, purified by affinity to VPg on CNBr-activated Sepharose 4B) (Grzela *et al.*, 2006).

Transgene mRNA was detected in five of six transgenic F2 plants; no transgene mRNA was detected in a plant from the F2_At_VPg3 line (Fig. 1 (1A)). Western blotting performed on protein extract from the transgenic plants with polyclonal antibodies to PVY VPg did not demonstrate any VPg expression (not shown).

Next, the expression and stability of the VPg transgene in F3 progeny was analysed. Seeds from the five F2_At_VPg lines that demonstrated VPg mRNA expression were collected and planted as before. The phenotype of all F3 plants was similar to wt *A. thaliana* plants. Out of the five F3 transgenic lines tested, in only two, F3_At_VPg4 and F3_At_VPg5, was the VPg transgene stable. All randomly selected plants from these two lines transcribed the transgene. In the remaining three lines, only one out of eight plants studied exhibited transgene expression (Fig. 1 (1B) and Table 1). As before, the obtained results were confirmed by PCR reaction with genomic DNA as a template. Finally, detection of VPg was attempted by Western blotting, but the results were again negative (not shown). To exclude the possibility that the lack of VPg expression at the protein level is due to incorrect gene sequence, the sequence of the *VPg* gene incorporated into the genome of transgenic *A. thaliana* was verified; in all F3 progeny plants tested with transgene mRNA expression the sequence of the *VPg* gene was correct. We did not attempt to estimate of the gene copy number as it is clear from numerous communications that there is rarely a correlation between the gene copy number and transgene mRNA abundance on one hand and plant resistance on the other (Zanek *et al.*, 2008; Febres *et al.*, 2008).

In the next step, experiments were initiated to determine whether in the presence of transgene the plants are resistant to infection with the cognate virus. Four F3 progeny plants, numbers 15, 22, 55 and 56, were selected to study their response to infection with PVY. Three plants (nos. 15, 22 and 55) demonstrated transgene expression while one (no. 56) turned out to be transgeneless and served as a negative control. Five-week-old plants were mechanically inoculated with PVY (strain NTN) on two or three leaves and grown in a growth chamber at 25/20°C under a long-day photoperiod (16 hours light and 8 hours darkness) at 75% relative humidity. Seven days post inoculation (dpi) all infected plants were symptom free. At 12 dpi plant no. 55 displayed yellow-green leaves while the others remained green (Table 2). The inoculated and non-inoculated leaves of each selected plants were tested for the presence of PVY coat protein (CP) mRNA, as CP is the most abundant product synthesized during PVY infection; the presence of CP and the CP mRNA indicates viral infection (Andrejeva *et al.*, 1999). Hence, expression of CP mRNA was tested by RT-PCR as above. Primers for this analysis were designed to amplify the CP cistron according to the sequence of PVY strain NTN (accession no. AJ889866). Actin mRNA was used as an internal control. CP mRNA was detected in two cases, F3_At_VPg1_15 and F3_At_VPg6_56, in the inoculated leaves only (Fig. 2). Of note, infection of the control plant (no. 56) with PVY did not result in CP expression in non-inoculated leaves.

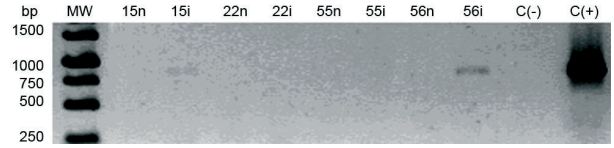

Figure 2. Detection of PVY coat protein (CP) mRNA in infected F3 progeny plants
n, non-inoculated leaves; i, inoculated leaves; C(+), positive control (Bluescript PVY-CP plasmid); C(–), negative control (no template); 15–56 selected plants (see Table 2); MW, DNA molecular weight markers.

DISCUSSION

The scarcity of obtained PVY VPg-transgenic seeds suggested that VPg introduced serious early defects in transformed plants, which precluded their growth. As a result, only few VPg-transgenic plants could be recovered. The transgenic lines grew almost normally and were morphologically undistinguishable from non-transgenic ones. They contained the correct *VPg* gene and synthesized presumably correct VPg mRNA, but no production of VPg protein could be detected by Western blotting. It is relevant that in the case of PVY P1–transgenic potato some plants produced P1 protein but resistance to PVY was not associated with detectable expression of P1 protein (Mäki-Valkama *et al.*, 2000).

Aphids can be controlled by using insecticides. However, since these chemicals are toxic and environmentally deleterious, engineering viral resistance in plants is an attractive approach. The first plants engineered for resistance to viruses were obtained by Powell-Abel *et al.* (1986). Henceforth, there have been numerous

Table 2. Analysis of VPg-trangenic F3 plants at 12 days post inoculation with PVY
Plants with transgene expression are marked in bold.

F3_At_VPg lines	Plant	Inoculated leaves	Non-inoculated leaves	Immunity against PVY
F3_At_VPg1	**15**	green	green	–
F3_At_VPg2	**22**	green	green	+
F3_At_VPg6	**55**	yellow-green	green	+
	56	green	green	–

attempts to generate virus resistance in transgenic plants based on expression of virus-derived genes or genome fragments, also for PVY (Lawson *et al.*, 1990; Vardi *et at.*, 1993; Pehu *et al.*, 1995; Baulcombe, 1996). In the case of PVY, some interesting results were obtained with potato plants transformed with a fragment of the coat protein (*CP*) gene, when approximately half of the transformed lines turned out to be resistant to challenge with PVY (Bukovinszki *et al.*, 2007). In addition, *NIb* gene-transformed potato plants acquired high resistance to PVY under laboratory conditions, but lost it under field conditions (Schubert *et al.*, 2004). Interestingly, transgenic potato plants obtained by transformation with PVY *P1* gene were immune to cognate virus but could be infected with a similar virus strain with above 96% sequence identity, suggesting that factors other than sequence homology might be important for immunity (Mäki-Valkama *et al.*, 2000). Indeed, an interaction of PVY CP protein with host DnaJ-like proteins was observed, and it appeared that tobacco plants deficient in those proteins showed a strong increase in PVY resistance (Hofius *et al.*, 2007); this might explain the anti-viral immunity of CP-transgenic tobacco plants (Bendahmane *et al.*, 2007).

Several attempts have been made to produce transgenic potyviruses-resistant plants using potyvirus VPg-containing sequences. It has been observed that transformation of *Nicotiana tabacum* plants with sequences corresponding to the fusion protein VPgPro (or NIa) of three potyviruses, *Tobacco vein mottling virus* (TVMV), *Tobacco etch virus* (TEV) and PVY, results in plants resistant to infection with the homologous virus (Fellers *et al.*, 1998). However, when *Nicotiana benthamiana* plants were transformed with *VPg* gene of *Potato virus A* (PVA) the transformed lines displayed three different phenotypes: (a) resistance to virus infection, (b) susceptibility, or (c) systemic infection followed by recovery of new leaves (Germundsson & Valkonen, 2006). Looking for the VPg effect on the suppression of RNA silencing, Germundson *et al.* (2007) found that two PVA VPg-transgenic lines, tt2 and tt7, readily expressed VPg protein, which did not interfere with the GFP transgene silencing (Germundsson *et al.*, 2007). When tobacco plants were transformed with three genes, each consisting of two different NIa coding regions (TEV NIa-PVY NIa, TEV NIa-TVMV NIa, and TVMV NIa-PVY NIa) and with a polyprotein, e.g., NIa–NIb–CP of TEV, of *Plum pox virus* (PPV) or of TVMV and CI–6K2–NIa or 6K2–NIa of TEV, the transformed lines displayed similarly three different phenotypes: (a) resistance to virus infection, (b) susceptibility, or (c) systemic infection followed by recovery of new leaves (Schaad *et al.*, 1996; Fellers *et al.*, 1998; Guo *et al.*, 1998; Maiti *et al.*, 1999). Those studies together demonstrated that only a small proportion of the transgenic lines exhibited resistance, and also that the level of resistance was lower following transformation with polyprotein-encoding constructs in comparison with sequences encoding single proteins (Maiti *et al.*, 1999; Germundsson *et al.*, 2007). Moreover, the resistance to viruses was most likely homology-dependent and did not protect the plants against infection with heterologous viruses (Savenkov & Valkonen, 2001).

The PTGS mechanism is based on sequence similarity between the transgene and the incoming virus. However, in several studies on transgenic plants another hypothetical mechanism is invoked, in which the establishment of plant virus resistance could depend on interactions of the transgene or of transgenic protein with plant machinery (Bendahmane *et al.*, 2007). In a thorough study of trangenic plants made with VPg of another potyvirus, *Potato virus A*, the authors did not report any difficulties in obtaining the VPg-trangenic plants (Germundsson *et al.*, 2007). Transgenic plants abundantly produced VPg mRNA and some plants did express detectable amounts of the VPg protein. One line (tt6) was resistant to infection, while another line (tt7) recovered from infection and was very resistant to superinfection (Germundsson & Valkonen, 2006). In addition, a PVX-based expression vector containing PVA *VPg* gene exhibited increased virulence, which could be attributed to a VPg interaction with the initiation translation factor of the host, eIF4E, affecting host protein synthesis. We have previously shown that PVY VPg protein has quite a high affinity to plant eIF4E (Grzela *et al.*, 2006). We think that the low yield of transgenic seeds as well as the low numbers of transgenic plants obtained stem from the fact that in seeds and plants able to produce PVY VPg, the disturbance of host protein synthesis was so severe that it resulted in early death. In contrast, in transgenic plants in which there was none or very little VPg production (below the threshold of detection by Western blotting) growth was not impaired. In conclusion, despite providing some immunity, the putative PVY VPg toxicity exerted at an early stage of transformed seed formation precludes its use for engineering pathogen-derived resistance.

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